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14. ABSTRACT

Rho-family GTPases are the major regulators of the actin and microtubule cytoskeleton. Regulators of the GTPases include GEFs and GAPs, which control activation and deactivation, respectively, of this family of molecular switches. This study explores the role of the GEF Tiam2 in breast cancer cell proliferation and invasion, which was initially discovered using a siRNA screen in MDA-MB-231 breast cancer cells. Protein expression studies are performed using a panel of cell lines, revealing that Tiam2 expression is upregulated in Ras mutant cells. Additionally, Tiam2 depletion causes MDA-MB-231 cells to form fewer colonies in soft agar colony formation assays. Work continues on this project to determine how Tiam2 contributes to breast cancer proliferation and invasion using both cell culture and mouse models.

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Introduction

Rho family GTPases, members of the Ras superfamily, are best known for their regulation of the actin and microtubule cytoskeleton.[1] Misregulation of Rho signaling pathways can contribute to oncogenesis through effects on cell proliferation, adhesion, and migration.[2] There is evidence that mutations or alterations in expression levels of the Rho GTPase regulatory proteins, guanine nucleotide exchange factors (GEFs) and activating proteins (GAPs), contribute to inappropriate activation of Rho-mediated signal transduction pathways.[3, 4] Tiam2, a guanine nucleotide exchange factor (GEF), was identified in a siRNA screen for defects in breast cancer cell migration and invasion. Relatively little is known about the role of Tiam2 in regulating breast cancer migration, invasion, and proliferation as compared to the closely related protein Tiam1.[5-8] Both *in vitro* and *in vivo* approaches will be used to characterize how Tiam2 signals through Rho GTPases to control invasion and growth of MDA-MB-231 breast cancer cells. This study will allow for identification of possible drug targets in addition to expanding the understanding of Tiam2's physiological role in tumor progression.

Body

A panel of cell lines was assembled that encompassed all many known breast cancer mutational statuses in addition to normal breast cell lines. Tiam1 and Tiam2 expression levels were compared [Figure 1]. Notably, expression of Tiam2 correlated with known Ras mutational status. Simple overexpression of mutant KRas V12 was insufficient to increase Tiam2 expression in HMEC and MCF-10a cells. Microarray expression data from the Oncomine database (www.oncomine.org) was used to examine Tiam2 expression in human tumors, but no differences were observed. Comparison of mRNA (by qPCR) and protein levels in MDA-MB-231 cells and derivatives showed that Tiam2 probes on the microarray chips were unable to specifically detect changes in Tiam2 levels (data not shown). To further dissect the link between mutant Ras expression and Tiam2 levels, MDA-MB-231 cells were treated with MEK and PI3K inhibitors [Figure 2]. Inhibition of the MEK using two inhibitors resulted in lower Tiam2 expression, while PI3K inhibition had no effect.

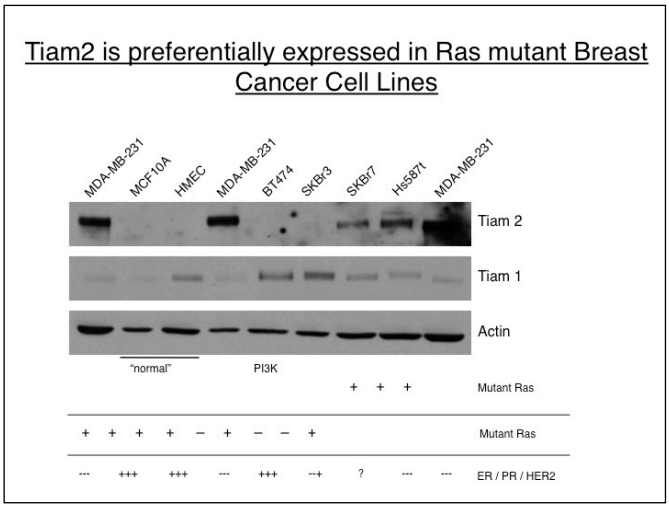

Figure 1: Expression patterns of Tiam1 and Tiam2 in a panel of Breast Cancer Cell Lines.

To rescue the invasion defect caused by siRNA depletion of Tiam2, a full-length human Tiam2 cDNA was obtained. Silent mutations were introduced into this cDNA, causing it to become resistant to the siRNA targeted against the endogenous protein. This resistant Tiam2 cDNA was PCR cloned into pBabe retroviral plasmid to create stable, puromycin resistant MDA-MB-231 derived cell lines. These cell lines were treated with siRNA targeted to Tiam2 in an attempt to rescue the invasion phenotype. While

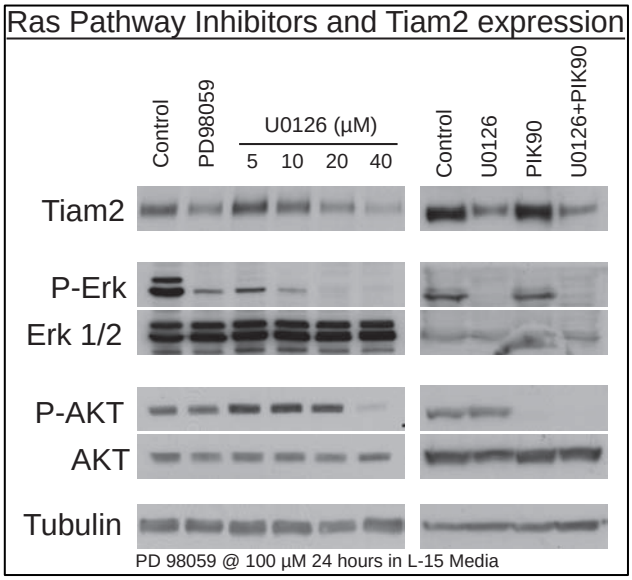

Figure 2: Inhibition of MEK and not PI3K results in decreased Tiam2 expression.

it was possible to rescue the protein expression, the derivative cells were unable to invade through the matrigel. To determine if MDA-MB-231 cells harbor an activating mutation in Tiam2 (which may explain the lack of rescue), mRNA from these cells was isolated, reverse transcribed, and sequenced. Two known SNPs were found, but no other mutations that might alter protein function. This creates a significant problem for further domain analysis if the phenotype is not rescued by expression of a siRNA-resistant Tiam2 cDNA, and will impede work on both the invasion and proliferation *in vitro* studies.

A panel of shRNA lentivirus plasmids were obtained and used to create stable Tiam2-depleted MDA-MB-231-derived cell lines. These lines were then grown in soft agar to determine if Tiam2 expression affected anchorage-independent growth. Two of the plasmids were able to stably reduce Tiam2 protein levels and also reduced the number of colonies in soft agar [Figure 2]. Cells with lower levels of Tiam2 showed a 15% increase in the proportion of cells in G1 as compared to control as determined by FACS. These early results encouraged further work using a xenograft mouse model of tumorigenesis.

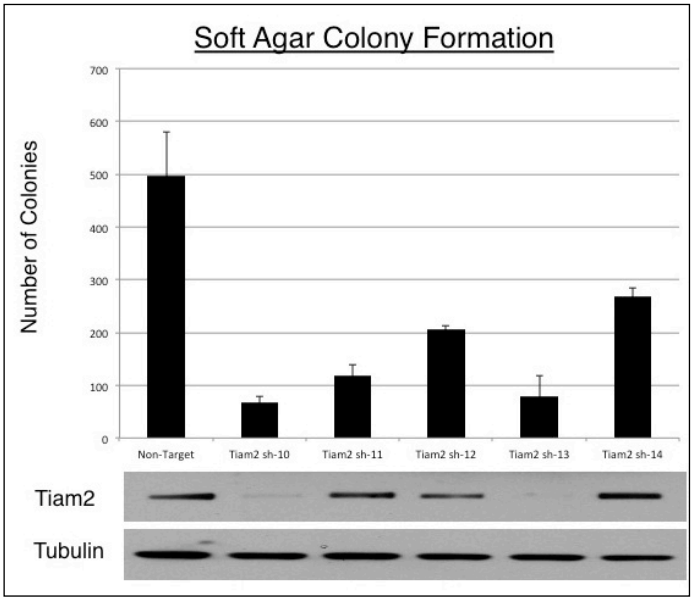

Figure 3: sh-10 and sh-13 deplete Tiam2 levels and most strongly inhibit the formation of colonies of MDA-MB-231 cells in soft agar.

The two hairpins used to successfully stably deplete Tiam2 in MDA-MB-231 colony formation assays were selected for use in a mouse tumorigenesis assay. With the assistance by the lab of Dr. Jacqueline Bromberg, NOD/SCID mice were orthotopically injected with these cells and tumor size was assayed after 10 weeks [Figure 4]. The individual hairpins affected *in vivo* tumor growth quite differently despite maintaining low levels of Tiam2. Derivative cell lines will be isolated from these mice also showed low levels of Tiam2 expression compared to control.

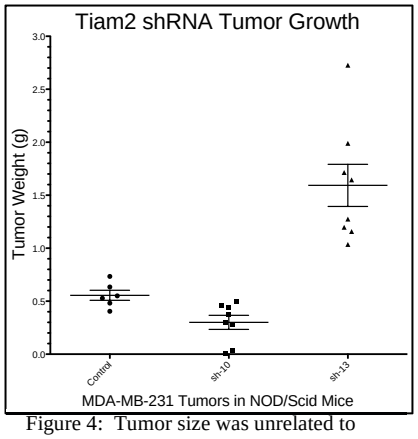

Figure 4: Tumor size was unrelated to Tiam2 expression levls.

Taken together, the effects of Tiam2 depletion by RNAi

seem to have no effect on invasion and inconclusive effects on tumor growth *in vivo*. Further work is underway to determine if Tiam2 levels affect GTPase activity both in MDA-MB-231 and Hs578t cells. Scratch assays will be performed to understand if there is a role for Tiam2 in migration rather than invasion in both cell types. Additionally, the level of GTPase activation will be studied along with other possible effector molecular targets [9].

Key Research Accomplishments

- Tiam2 expression is up-regulated in Ras mutant cancer cell lines.
- Inhibition of MEK but not PI3K signaling decreases Tiam2 protein level.
- Tiam2 depletion inhibits the formation and growth of colonies in soft agar.
- Creation of MDA-MB-231 lines stably expressing siRNA-resistant Tiam2.
- Creation of MDA-MB-231 lines stably expressing shRNA depleting Tiam2 levels.

Reportable Outcomes

- Poster Presentation: Jeffery R. Smith and Alan Hall; "The Role of Rho GTPases in Breast Cancer Invasion and Proliferation". 2011 Era of Hope Meeting in Orlando, FL.

Conclusion

This study illustrates the importance of careful analysis of expression levels independent of microarray data. Increased expression of Tiam2 protein and mRNA in Ras-mutant breast cancer cell lines is unexpected and overlooked by previous work. Further studies should explore the link between these expression patterns as well as transcriptional regulation of protein expression during metastasis. Invasion studies, while slowed by the inability of Tiam2 overexpression to rescue invasion inhibition, still imply that Tiam2 could play a role in cell motility. Further work using animal models is currently underway to further elucidate the importance of Tiam2 in tumor formation, progression, and metastasis. This work will add insight into the molecular mechanisms by which cancer cells invade into surrounding tissue and eventually result in death.

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Appendices

None.